Oxidation of 1-*O*-(Alk-1-enyl)-2,3-Di-*O*-Acylglycerols: Models for Plasmalogen Oxidation[1]

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Alk-1-enyl diacyl glycerides, model compounds for plasmalogen lipids, were synthesized for use as substrates in oxidation studies. The neutral plasmalogen glycerides prepared included 1-*O*-(hexadec-1-enyl)-2,3-di-*O*-stearoylglycerol (*4a*) and 1-*O*-(hexadec-1-enyl)-2-*O*-linoleoyl-3-*O*-stearoylglycerol (*4b*). Oxidative disappearance of these glycerides was followed directly by high performance liquid chromatography (HPLC). The extent of oxidation of the side chains of these glycerides, i.e. the alkenyl ether and fatty acyl functions, was monitored by conversion of the side chains to dimethylacetal and methyl ester by methanolysis, followed by subsequent gas chromatography (GC) of the methanolysis products. Both analytical approaches show that the alkenyl ether function of neat *4a* oxidizes more slowly than neat ethyl linoleate. However, the rate of alkenyl ether loss from *4a* is accelerated in the presence of added ethyl linoleate. Moreover, when the linoleoyl group is incorporated into the 2-position of the alkenyl glyceride, as in *4b*, the rate of loss of the alkenyl group was shown to be similar to the rate of loss of the linoleoyl group. These results suggest that oxidation of plasmalogen glycerides should not be ignored as a factor that contributes to the oxidative instability of animal tissue.
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In muscle and other animal tissue, phosphatides are not confined just to the diacyl variety. Substantial amounts of the long-chain groups at the glycerol *sn*-1-position are in the form of alkyl ethers, and as much as a third of the choline glycerophosphatides and two-thirds of the ethanolamine phosphatides are plasmalogens, i.e. *sn*-1-(alk-1-enyl) ethers (1). Studies of lipid oxidation in foods typically are focused on the role of autoxidation of polyunsaturated acyl groups in phosphatides (generally at the *sn*-2 position), especially with regard to the course of such oxidation in the development of rancidity in meat (2–5). On the other hand, only very recently has attention been focused on the autoxidation of the double bond in plasmalogens as a potential contributor to such rancidity (6). In the latter report, it was shown that a model enol ether, ethyl hexadec-1-enyl ether, was prone to in vitro autoxidation, especially in the presence of polyunsaturated esters. Other studies have shown that natural plasmalogens are subject to in vivo peroxidation by enzymatic systems (7–9) and that alk-1-enyl ethers are subject to attack by singlet oxygen (10,11). This study was undertaken to investigate whether the alk-1-enyl functionality of neutral plasmalogen glycerides undergoes autoxidation as do simple vinyl ethers and to assess the effect of polyunsaturated acyl functionality, both inter- and intramolecular, on the course of such autoxidation.

MATERIALS AND METHODS

Hexadecanal was prepared from hexadecanol (Aldrich Chemical Co., Milwaukee, WI) by oxidation using dimethylsulfoxide (DMSO) and dicyclohexylcarbodiimide (Sigma Chemical Co., St. Louis, MO) according to the method of Fenselau and Moffatt (12). Glycerol-1,2-carbonate was prepared by the ester interchange of D,L-1-*O*-benzylglycerol (Sigma Chemical Co.) and diethyl carbonate (Aldrich Chemical Co.), followed by removal of the benzyl group by hydrogenolysis with palladium on charcoal following the method of Cunningham and Gigg (13). Stearoyl and linoleoyl chloride were obtained from their corresponding acids by reaction with oxaloyl chloride (Aldrich Chemical Co.) by the method of Bosshard et al. (14).

Thin layer chromatography. TLC was carried out on plates of Silica Gel G (250μ) obtained from Analtech (Newark, DE). Visualization of acetal and alk-1-enyl ether products was accomplished by fuchsin-bisulfite spray after exposure of the plates to concentrated hydrochloric acid vapors for one min; aldehydes so liberated were detected as purple to red spots. All products were visualized by spraying the plates with 6% copper sulfate in 50% phosphoric acid, followed by charring.

Gas chromatography. GC was conducted on a Sigma-3 gas chromatograph (Perkin-Elmer, Norwalk, CT) equipped with a capillary injector and flame ionization detector (FID). Separations were obtained using a fused silica wall-coated open tubular column 1.2 m × 0.2 mm i.d. (Hewlett-Packard, Avondale, PA) coated with 0.33μ thick, cross-linked methyl silicone. Determinations were made using helium as a carrier gas (1 ml/min) with a split ratio of 80:1 and the following oven program: seven min at 155 C, then 5 C/min to 200 C. Ethyl heneicosanoate (21:0) served as internal standard because it did not undergo oxidation under the experimental conditions. Signal analysis was accomplished by routing the detector output to the integrating terminal of a Hewlett-Packard 5880A gas chromatograph. A GC trace that shows the separation of the components of interest is shown in Figure 1.

High performance liquid chromatography. HPLC was carried out with a system consisting of a Beckman (Fullerton, CA) Model 110 A solvent delivery module equipped with a Tracor (Austin, TX) Model 945 (FID, an Altex Model 210 loop injector and a 20 μl loop. The HPLC column used was an Altex Ultrasphere-ODS, 5μ particle size, stainless steel column, 4.6 mm i.d. × 25 cm. The FID output was routed to a Hewlett-Packard Model 3390 integrator to determine retention times and peak areas. Triheptadecanoin (NuChek Prep, Elysian, MN) served as internal standard for the HPLC separations shown in Figure 2. Samples were eluted with methylene chloride/

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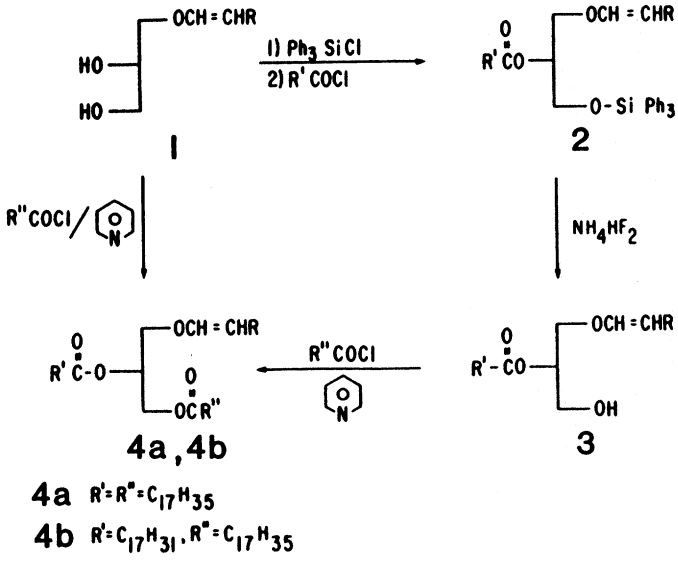
Abbreviations: DMA, dimethyl acetal of hexadecanal; DMSO, dimethylsulfoxide; FID, flame ionization detector; GC, gas chromatography; HPLC, high performance liquid chromatography; ISTD, internal standard; TLC, thin layer chromatography.

acetonitrile (55:45, v/v) at a flow rate of 1 ml/min. Samples were dissolved in the eluent and 20 μl injected.

Synthesis of cis/trans-*1-O-(hexadec-1-enyl)glycerol.* Hexadecanal (20 mmol) was converted to its bis-(2,3-carbonylglycerol) acetal by reaction with glycerol-1,2-carbonate (50 mmol) in the presence of methanesulfonic acid (1 mmol) in 30 ml of benzene/N,N-dimethylformamide (99:1) heated at reflux using a modification of the procedure of Chebyshev et al. (15). The acetal was purified by recrystallization from ether, yield 30%, m.p. 75–79 C. TLC of this product, R_f 0.21, CH_2Cl_2 solvent, showed only one component. Reaction of the acetal with phosphorus pentachloride according to the method of Chebyshev et al. (16) and then elimination of hydrogen chloride with triethylamine, according to the method of Gigg and Gigg (17), gave 1-*O*-(hexadec-1-enyl)glycerol-2,3-carbonate. The latter compound was purified by flash chromatography on silica gel (grade 60, 230–400 mesh, Aldrich Chemical Co.) using ether as eluent, TLC R_f 0.76, ether as eluent. Saponification of 1-*O*-(hexadec-1-enyl)glycerol-2,3-carbonate with methanolic potassium hydroxide (17) gave 1-*O*-(hexadec-1-enyl)glycerol (1, Scheme 1) in 68% yield, m.p. 43–44 C recrystallized from isooctane, TLC R_f 0.64 for *trans* isomer, 0.79 for *cis* isomer, eluent ether.

Synthesis of cis/trans-*1-O-(hexadec-1-enyl)-2,3-di-O-stearoylglycerol (4a).* To a solution of 1-*O*-(hexadec-1-enyl)-glycerol 1 (2 mmol) in carbon tetrachloride (10 ml) was added pyridine (10 ml) and stearoyl chloride (4.4 mmol). The reaction mixture was heated at 40 C for one hr under nitrogen, cooled to ambient temperature and filtered through neutral alumina (20 g, 80–200 mesh, Fisher Scientific Co., Fairlawn, NJ) to remove the precipitated pyridinium hydrochloride. The column was washed with an additional 40 ml of carbon tetrachloride, and the eluates were combined. The solvents were removed in vacuo and the residue chromatographed on silica gel (10 g). Elution with methylene chloride/hexane (3:1) gave compound 4a in 55% yield, R_f 0.90 using methylene chloride/hexane (3:1), m.p. 51–52 C after recrystallization from hexane.



SCHEME 1

Synthesis of cis/trans-*1-O-(hexadec-1-enyl)-2-O-linoleoyl-3-O-stearoylglycerol (4b).* To a solution of 1-(*O*-hexadec-1-enyl)glycerol 1 (2 mmol) in carbon tetrachloride/pyridine (20 ml, 1:1) was added chlorotriphenylsilane (Aldrich Chemical Co.) (2 mmol in 5 ml of carbon tetrachloride). The resulting mixture was stirred at ambient temperature for one hr. TLC of the reaction mixture showed completeness of reaction. Linoleoyl chloride (2 mmol) was added to this solution, and the reaction mixture was heated to 40 C for one hr. The mixture was filtered through a column of neutral alumina (5 g), and the column was washed with chloroform (40 ml). The column eluates were combined, the solvents removed in vacuo, and the residue was chromatographed on silica (20 g) using methylene chloride/hexane (3:1) as eluent. TLC of fractions showed product 2 (Scheme 1) as a mixture of *cis* (R_f 0.62) and *trans* (R_f 0.54) isomers, solvent hexane/ether (4:1), yield 60%. A solution of 2 (1 mmol) in acetone (6 ml) and pyridine (0.2 ml) was heated at reflux for one hr in the presence of ammonium hydrogen fluoride following the procedure of Chebyshev et al. (18). Chromatography on silica gel and elution with methylene chloride/hexane (3:1) gave pure 3, Scheme 1 as a viscous oil, R_f 0.24, hexane/ether (3:1) in 42% yield. Reaction of 3 (1 mmol) with stearoyl chloride (1.1 mol) in carbon tetrachloride/pyridine (1:1) as described above gave compound 4b, Scheme 1, in 72% yield after chromatography on silica gel using methylene chloride/hexane (3:1) as eluent. Purified 4b had an R_f of 0.77, hexane/ethyl acetate (3:1).

Oxidation procedure. Carefully weighed mixtures of either alk-1-enyl ether 4a or 4b, the appropriate internal standard and ethyl linoleate in the amounts listed in Table 1 were prepared in 500 μl of pentane. Aliquots of 50 μl then were transferred to each of nine serum vials (120 ml capacity; nominally 100 ml), the pentane removed in vacuo, the vacuum broken with air and the vials sealed with aluminum caps fitted with PTFE-lined butyl rubber septa (Perkin-Elmer). The sealed vials were immersed in a constant temperature water bath at 86 C. Vials were withdrawn at half-hour intervals and allowed to cool to room temperature. Reaction mixtures then were analyzed by either of the following procedures. HPLC analysis: the inside wall of the cooled vial was washed down with 600 μl

TABLE 1

Composition of Reaction Mixtures Prior to Oxidation, in μmol[a]

Component	Run 1[b]	Run 2[b]	Run 3[c]	Run 4[c]	Run 5[c]	Run 6[b]
4a	15.7	14.5	14.4	14.4		
4b					13.5	10.9
18:2[d]		15.2		16.2		
21:0[d,e]	13.3	16.9				13.3
Trihepta-decanoin[e]			14.1	10.6	10.8	

[a]Oxidation carried out under oxygen (ca. 1100 μmol), in sealed vials of 120 ml capacity.
[b]Analysis by GC.
[c]Analysis by HPLC.
[d]As ethyl ester.
[e]Internal standard.

of methylene chloride and 400 μl of acetonitrile, the resulting solution was transferred by means of a Pasteur pipette to a 1-ml serum vial, and 20 μl was injected onto the HPLC column. GC analysis: to each cooled vial 2 ml of 1% H_2SO_4 in methanol was added. The vial was heated on a steam bath for 15 min with occasional shaking and then cooled to room temperature. Saturated aqueous ammonium acetate (4 ml) added, and the resulting mixture was extracted with hexane (2 ml × 2). The combined extracts were dried over anhydrous Na_2SO_4, and 3 μl of the dried extract were injected onto the GC column for analysis.

RESULTS AND DISCUSSION

Synthesis of model neutral plasmalogens. The structures for the model 1-*O*-(alk-1-enyl) glycerides (neutral plasmalogens) synthesized for this study are shown in Scheme 1. Compound **4a**, 1-*O*-(hexadec-1-enyl)-2,3-di-*O*-stearoylglycerol, was prepared directly from 1-*O*-(hexadec-1-enyl)glycerol (**1**) by reaction with two equivalents of stearoyl chloride. Compound **4a** served as a model for studying the 1-*O*-(alk-1-enyl) functionality, found in plasmalogens, for its susceptibility to oxidation. The stearoyl residues of **4a** were not anticipated to be prone to oxidation because we had previously shown that saturated fatty esters, in particular ethyl stearate, are stable under the experimental conditions used in this study (6).

1-*O*-(Hexadec-1-enyl)-2-*O*-linoleoyl-3-*O*-stearoylglycerol, **4b** (Scheme 1), was prepared as a model neutral plasmalogen having internal polyunsaturation. Compound **4b** was obtained from compound **1** by protection of the 3-*O*-position of glycerol with chlorotriphenylsilane to yield compound **2** (Scheme 1). The latter compound, after acylation with linoleoyl chloride and subsequent removal of the silyl protective group, yielded compound **3** (Scheme 1). Reaction of **3** with stearoyl chloride gave the desired compound **4b**. Placement of the linoleoyl residue at the 2-*O*-position of the glycerol backbone of **4b** was opted because this is the position in which unsaturated acyl residues typically are located in naturally occurring neutral and polar plasmalogens (19).

Monitoring techniques for following autoxidation. Table 1 lists the amounts of each substrate used in the oxidation experiments described herein. Two complementary analytical procedures, HPLC and GC, were used in monitoring the oxidative disappearance of the neutral plasmalogens **4a** and **4b**. For Runs 1, 2 and 6, Table 1, substrate depletion was followed by GC using methyl heneicosanoate as an internal standard. In this procedure, the plasmalogens were converted to dimethyacetals and methyl esters with methanol containing 1% sulfuric acid and brief heating at 100 C (Fig. 1). With this procedure, we were able to follow not only the loss of 1-*O*-(hexadec-1-enyl) functionality but also the loss of linoleoyl functionality in **4b**, the latter of which is subject to oxidation under the experimental conditions. Runs 3, 4 and 5, Table 1, were monitored by following the direct disappearance of plasmalogens **4a** and **4b** by HPLC coupled with a FID. Triheptadecanoin was used as internal standard in these experiments. Though loss of linoleoyl functionality in **4b** could not be followed, the loss of added ethyl linoleate to **4a** could be monitored (Fig. 2).

Autoxidation results. The results of the oxidative disappearance of **4a** and **4b** are presented graphically (Figs. 3–6) for a series of experiments carried out at 86 C, a temperature that allowed for a measurable rate of substrate disappearance over a five-hr reaction period. The data are graphed in normalized fashion to show the percentage of remaining components at specified reaction times. All experiments in Table 1 were carried out in sealed vials of sufficient volume (120 ml) to ensure an excess of oxygen (1100 μmol) to eliminate oxygen content as a reaction variable. For oxidation of **4a**, the combined results obtained from Runs 1 and 2 are shown graphically in Figure 3 and combined results from Runs 3 and 4 in Figure 4. For oxidation of **4b**, results from Run 5 are shown in Figure 5 and Run 6 in Figure 6.

Oxidation of 4a. Run 1 (Fig. 3) shows the GC results obtained when compound **4a** (Scheme 1, devoid of polyunsaturation) was subjected to the oxidation conditions employed in these studies. Over the 4.5-hr reaction time of this experiment, a slow but measurable disappearance

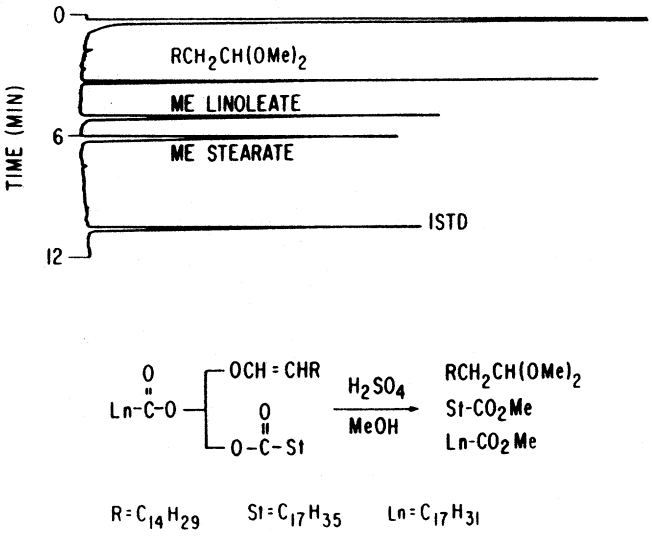


FIG. 1. GC separation of the ethanolysis products of plasmalogen glyceride *4b*; internal standard (ISTD) = methyl heneicosanoate. Conditions as in Materials and Methods.

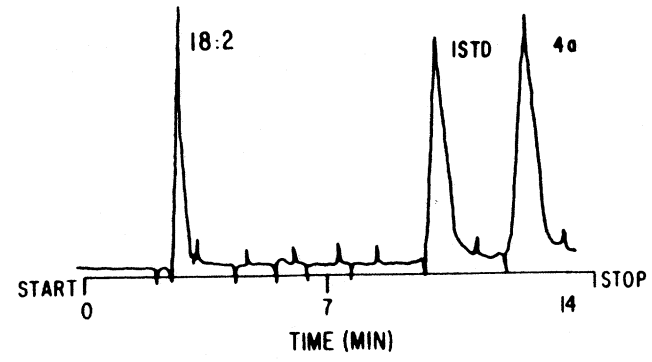


FIG. 2. HPLC of plasmalogen glyceride *4a*, ethyl linoleate (18:2) and triheptadecanoin (internal standard, ISTD). Conditions as in Materials and Methods.

of the 1-*O*-(alk-1-enyl) functionality of 4a was observed; by the end of the reaction period ca. 15% of the original amount of 4a had reacted. This run, which used ethyl heneicosanoate as internal standard, also showed that the stearoyl residues of 4a were unreactive under the conditions employed, a result in agreement with our previous work (6) with ethyl stearate.

The oxidative nature of the disappearance of 4a was established by repeating Run 1 with 0.1% antioxidant, w/w (Tenox 5; Kodak, Rochester, NY). The presence of antioxidant led to complete inhibition of decomposition of 4a over the five-hr reaction time. In addition, reactions failed to proceed when nitrogen was substituted for air in the vial headspace. Thus, loss of alk-1-enyl functionality in 4a cannot be attributed to hydrolysis by trace amounts of acid or water.

Also shown in Figure 3 are the results of Run 2, the oxidation of 4a in the presence of added ethyl linoleate. It is seen that linoleate oxidizes at a much faster rate than does 4a; however, the disappearance of 4a is accelerated in the presence of ethyl linoleate in that by the end of Run 2 almost twice the amount of 4a had reacted compared with Run 1. This result implied that there is accelerated oxidation of the 1-*O*-(alk-1-enyl) functionality of 4a by the oxidation products of linoleate.

In Run 3, Figure 4, the course of the reaction was followed by HPLC, which allowed for the direct measurement of disappearance of 4a, rather than its methanolysis

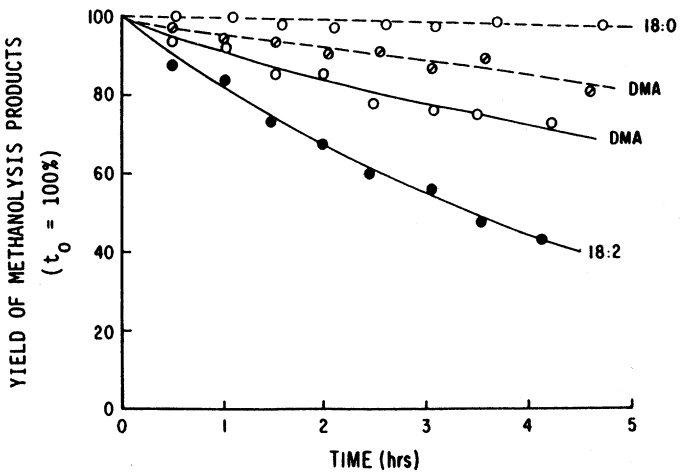


FIG. 3. Course of reaction at 86 C of *4a* as followed by GC of methanolysis products after treatment of oxidized 4a with H_2SO_4/methanol. – – – –, Run 1 (oxidation of *4a* in absence of ethyl linoleate); ——, Run 2 (oxidation of *4a* in presence of ethyl linoleate). 18:0, methyl stearate; DMA, dimethyl acetal of hexadecanal; 18:2, methyl linoleate; internal standard, methyl heneicosanoate; t_o denotes starting time of oxidation reaction.

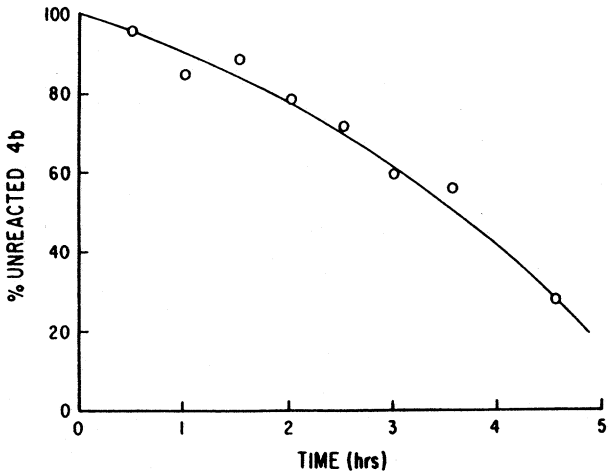


FIG. 5. Course of reaction at 86 C of *4b* (Run 5) as followed by HPLC. Internal standard, triheptadecanoin.

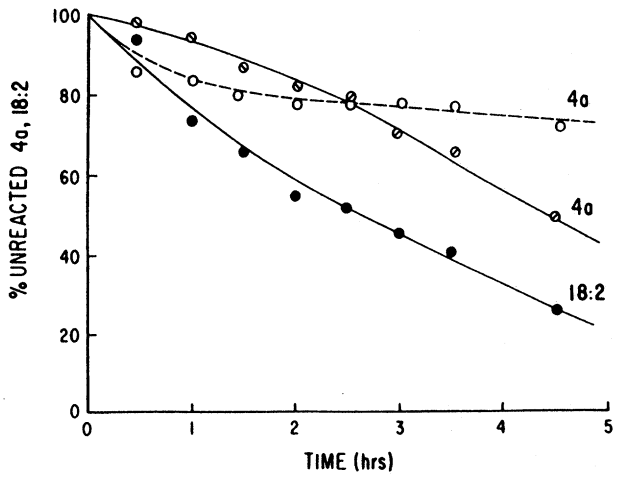


FIG. 4. Course of reaction at 86 C of *4a* as followed by HPLC. – – – –, Run 3 (oxidation of *4a* in absence of ethyl linoleate); ——, Run 4 (oxidation of *4a* in presence of ethyl linoleate). 18:2, ethyl linoleate; internal standard, triheptadecanoin.

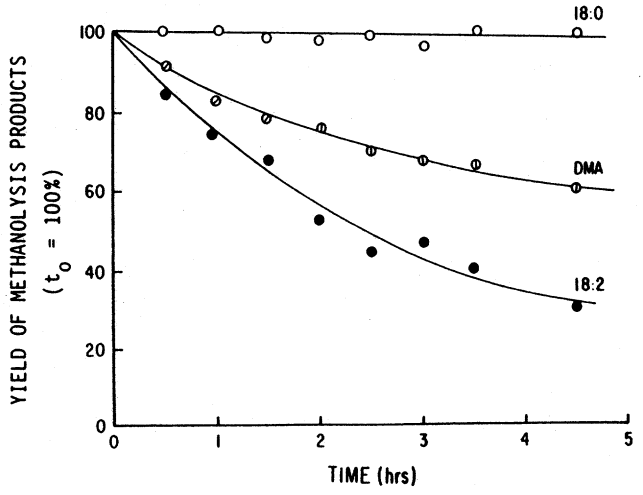


FIG. 6. Course of reaction at 86 C of *4b* (Run 6) as followed by GC of methanolysis products. 18:0, methyl stearate; 18:2, methyl linoleate; DMA, dimethyl acetal of hexadecanal; internal standard, methyl heneicosanoate; t_o denotes starting time of oxidation reaction.

products as in Run 1. In this manner, it was possible to compare the extent of oxidation on each side on the ether structure, that which occurred on the unsaturated (alk-1-enyl) side of the ether vs the saturated (CH_2 of glycerol backbone) side. A comparison of these two runs (1 and 3) shows a 20% disappearance of **4a** in Run 3 vs a 15% loss in Run 1. Accordingly, it is estimated that ca. 75% of the oxidation of **4a** occurs on the unsaturated side of the molecule, and 25% occurs on the saturated side. This result was in agreement with our earlier findings on the oxidation of the simple enol ether model, ethyl hexadec-1-enyl ether.

Run 4, Figure 4, is a repeat of Run 2, oxidation of **4a** in the presence of ethyl linoleate. Run 4 was followed by HPLC instead of by GC. As noted in Run 2, there was an accelerated depletion of **4a** in the presence of added ethyl linoleate; a 35% loss of **4a** was observed in Run 4. The initial rate of loss of **4a** was diminished in the presence of added linoleate, but as linoleate oxidation proceeded the rate of loss of **4a** increased even beyond that of Run 2 (Fig. 3). This result implied that the initial oxidation of linoleate retarded the loss of **4a**; however, as linoleate oxidation proceeded, the accumulation of its oxidation products accelerated the loss of **4a**. This indicated an interaction of linoleate oxidation products, presumably hydroperoxides (20), with the 1-*O*-(alk-1-enyl) function of **4a**. To test this hypothesis, we subjected ethyl linoleate alone to the oxidation conditions and obtained a depletion curve similar to that shown in Figure 4. HPLC of the resulting reaction mixtures showed, in addition to residual ethyl linoleate, a peak eluting earlier in the chromatogram, which could be ascribed to linoleate hydroperoxides (20). Unreacted **4a** in a nitrogen atmosphere then was added to this oxidation mixture, and this mixture was heated at 86 C for one hr. HPLC analysis of the latter mixture showed a loss of ca. 7% of **4a**, indicating a small but significant interaction of the alk-1-enyl function of **4a** with preformed linoleate oxidation products. Presumably, the greater loss of **4a** in the presence of oxidizing linoleate arises from a stronger intermolecular interaction of **4a** with linoleate oxidation intermediates, hydroperoxy radicals, than with linoleate oxidation products, hydroperoxides.

Oxidation of 4b. To assess the effect of intramolecular interaction of linoleate oxidation products with the alk-1-enyl ether functionality of plasmalogens, experiments were carried out using the internally polyunsaturated compound **4b**, Scheme 1, as substrate. Run 5, Figure 5, shows the results obtained when the oxidation of **4b** was followed by HPLC. From this run, it is seen that there is a rapid oxidation of substrate **4b** to the extent that the depletion curve resembles that of linoleate itself. This result was not totally unexpected because compound **4b** contained both of the oxidizable functional groups within its structure. A better picture of the course of oxidation of **4b** was obtained when the reaction was followed by GC, since with this technique it was possible to monitor the loss of alk-1-enyl and linoleoyl functionalities of **4b** separately. As the data show (Run 6, Fig. 6), loss of linoleoyl group amounted to ca. 60%. This confirmed the faster oxidation of linoleoyl residue over the alk-1-enyl functionality. Nevertheless, loss of alk-1-enyl group of **4b** was comparable to or exceeded that observed when **4a** was oxidized in presence of added ethyl linoleate, thus confirming a strong intramolecular interaction between the two oxidizing species. Finally, a comparison of the total oxidation loss of the two functional groups of **4b**, Figure 6, with oxidation loss of intact **4b**, Figure 5, indicated that both functional groups within **4b** are oxidized simultaneously.

From the above series of experiments, it has been demonstrated that the alk-1-enyl functionality of plasmalogens is subject to oxidation under relatively mild oxidizing conditions. More importantly, the data suggest that more easily oxidized polyunsaturated esters accelerate autoxidation of the alk-1-enyl ether functionality of plasmalogens. This acceleration of oxidation by polyunsaturated acyl residues has been shown to occur by either an inter- or intramolecular process. This is highly significant because natural plasmalogens typically also are highly polyunsaturated. Accordingly, the results suggest that oxidation of the alk-1-enyl ether functionality of plasmalogens should not be ignored as a factor that contributes to the oxidative instability of animal tissue or the development of rancidity in meat products.

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